Sequence effect on incision by (A)BC excinuclease of 4NQO adducts and UV photoproducts

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ABSTRACT

Nucleotide excision repair in *Escherichia coli* is initiated by (A)BC excinuclease, an enzyme which incises DNA on both sides of bulky adducts and removes the damaged nucleotide as a 12 – 13 base long oligomer. The incision pattern of the enzyme was examined using DNA modified by 4-nitroquinoline 1-oxide (4NQ0) and UV light. Similar to the cleavage pattern of UV photoproducts and other bulky adducts, the enzyme incises the 8th phosphodiester bond 5′ and 5th phosphodiester bond 3′ to the 4NQO-modified base, primarily guanine. The extent of DNA damage by these agents was determined using techniques which quantitatively cleave the DNA or stop at the site of the adduct. By comparison of the intensity of gel bands created by (A)BC excinuclease and the specific cleavage at the damaged site, the efficiency of (A)BC excinuclease incision at 13 different 4NQO-induced adducts and 13 different photoproducts was determined by densitometric scanning. In general, incisions made at 4NQO-induced adducts are proportional to the extent of damage, though the efficiency of cutting throughout the sequence tested varies from 25 to 75%. Incisions made at pyrimidine dimers are less efficient than at 4NQO-adducts, ranging from 13 to 65% incision relative to modification, though most are around 50%. The two (6 – 4) photoproducts within the region tested are incised more efficiently than any pyrimidine dimer.

INTRODUCTION

The removal of a wide variety of chemically and physically induced DNA lesions in *Escherichia coli* is mediated by the general pathway of nucleotide excision repair (1). The enzyme responsible for the incision and excision steps of this process is (A)BC excinuclease (2). It has been known for several years that the enzyme does not recognize and incise all types of damaged substrates with equal efficiency. For instance, pyrimidine dimers are not repaired as efficiently as (6–4) photoproducts or many chemically-induced lesions (3,4). It has been generally assumed, however, that multiple lesions consisting of the same adduct in a given DNA fragment are repaired with near equal efficiency (5). We have previously observed that with (6–4) photoproducts lesions are repaired with near equal efficiency (about 50%), suggesting that ABC excinuclease incises at these adducts in a sequence-independent manner (3). This may not be the case for some lesions. It is known that the orientation of a psoralen crosslink can determine the efficiency of repair (6,7). In another case, the DNA context in which the lesion resides greatly affected repair even over a very short distance as shown recently with acetylaminofluorene-induced adducts (8). The state of supercoiling was shown to affect the repair of N-methylmitomycin C-DNA monoadducts and interstrand crosslinks (9). To determine whether such variability in efficiencies of incision is a general phenomenon for (A)BC excinuclease, we have examined multiple sites on DNA fragments for their susceptibility to cleavage by the enzyme and compared the level of incision to the extent of DNA damage. We have chosen two different damaging agents for these experiments: a chemical agent, 4-nitroquinoline-1-oxide and a physical agent, UV light.

The repair of UV photoproducts has been extensively studied with this enzyme (1,2), although a systematic study of the efficiency of incision of pyrimidine dimers has not yet been performed on a sequence level. Using T4 DNA polymerase under conditions where the 3′ to 5′ exonuclease activity quantitatively degrades a DNA fragment immediately up to a photoproduct, the extent of UV damage in a given fragment can be accurately measured (10,11). We have compared this reaction with the cleavage products of the same DNA treated with (A)BC excinuclease to measure the efficiency of incision by the enzyme at multiple pyrimidine dimers and (6–4) photoproduct sites.

The potent carcinogen 4-nitroquinoline 1-oxide behaves very similarly to UV light in several repair systems (12–16). In *E. coli*, it has been shown genetically that the UvrABC system is responsible for repair of the 4NQO-induced lesion (1), though this has not been previously demonstrated *in vitro* with the

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purified enzyme. This is because, until recently, no method was available for preparing DNA substrate containing the 4NQO-induced 4AQO adduct. 4NQO is normally activated in the cell to the form of an acyl-4AQO which in turn reacts with DNA at primarily guanine bases, and to a lesser extent at adenine bases (17). This active form has now been prepared synthetically so that purified, end-labeled DNA fragments can be modified and tested with repair enzymes. It has been shown that the activated monoacetyl- and diacetyl-4HAQO forms of 4NQO react with DNA *in vitro* to form the same products produced *in vivo* (17–19). The extent of modification at all C8-4AQO adducts within a given fragment was determined by alkaline hydrolysis, which quantitatively cleaves the DNA at the lesion (19). The same modified DNA was also treated with (A)BC excinuclease and the efficiency of repair determined by densitometric scanning of alkaline and enzyme generated bands on autoradiograms. 4NQO-induced adducts are repaired more efficiently than pyrimidine dimers in general, most being repaired with greater than 60% efficiency.

MATERIALS AND METHODS

Materials

The UvrA, UvrB, and UvrC proteins were purified as described (20). pBR322 DNA was propagated in AB2487 and purified by sarkosyl lysis and two successive CsCl-ethidium bromide density gradient centrifugations. T4 DNA polymerase, T4 polynucleotide kinase and Klenow fragment were obtained from Boehringer Mannheim Biochemicals. 4NQO was obtained from Sigma. Monoacetyl- and diacetyl-4HAQO were prepared as described (18).

Methods

Preparation of Substrates. The (EcoR I-BamH I)$_{381}$ fragment of pBR322 was purified by electrophoresis on a 5% polyacrylamide gel, followed by electroelution. To prepare a 5′-end labeled substrate for analysis of incision at 4NQO-induced adducts, the purified fragment was treated with bacterial alkaline phosphatase and then T4 polynucleotide kinase using [γ-^{32}P]ATP. The kinased fragment was then cleaved with Hae III restriction endonuclease and the [EcoR I-Hae III]$_{174}$ fragment was gel purified and dialyzed against TEN-7.4 (10 mM, Tris-HCl, pH 7.4; 1 mM EDTA, 10 mM NaCl). To analyze 3′ incisions on the same strand as that used for 5′ incisions, a DNA fragment labeled at the 3′ terminus was prepared by first treating the products with Klenow fragment in the presence of [α-^{32}P]dCTP and excess cold dGTP, dATP and TTP. The (EcoR I-Mae III)$_{131}$ fragment, labeled only at the Mae III end, was gel purified and dialyzed against TEN-7.4.

The purified end-labeled fragments were then reacted with monoacetyl-4HAQO as follows. Four mg dithiothreitol, dissolved in 125 μl DMSO were mixed with 5 mg of diacetyl-4HAQO, also dissolved in 125 μl DMSO and allowed to sit 15 minutes at room temperature to form monoacetyl-4HAQO. Ten μl of end-labeled DNA (approximately 200 ng) was added to 190 μl of sodium citrate buffer (2 mM, pH 7.0) and 5 μl of the monoacetyl-4HAQO solution. After a 15 minute incubation at 37°C, 20 μl of 3M sodium acetate was added, and the sample was extracted three times with ether, precipitated with ethanol and finally resuspended in TEN-7.4. This treatment results in production of less than one C8-4AQO adduct per strand as determined by piperidine cleavage of modified DNA.

A 5′-end labeled fragment with UV photoproducts was prepared as follows for analysis of incisions at UV photoproducts. The (EcoR I-Hae III)$_{175}$ fragment of pBR322 was 5′-terminally labeled with polynucleotide kinase using [γ-^{32}P]ATP. The fragment was then digested with Hind III and the (Hae III-Hind III)$_{146}$ fragment 5′ labeled at the Hae III terminus was gel purified.

The purified DNA was irradiated with 500 J m^{-2}. This fluence produces on the average 1.5 pyrimidine dimers and 0.1–0.2 (6–4) photoproducts per fragment (21).

(A)BC exinuclease reactions and analysis of reaction products. Modified end-labeled DNA fragments were incubated with (A)BC excinuclease in a 40-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 50 mM HCl, 10 mM $MgCl_2$, 2 mM ATP, 10 mM dithiothreitol, 800 μg/ml bovine serum albumin, 50 ng of end-labeled DNA, and 100 ng each of the UvrA, UvrB, and UvrC proteins. The reaction mixture was incubated for 15 minutes at 37°C. The DNA was then deproteinized by phenol extraction and lyophilized. The sample was dissolved in formamide plus dyes, heated at 90°C for 1 minute, and analyzed on 8% sequencing gels alongside the Maxam and Gilbert (22) sequence ladders of the same fragment.

The efficiency of incision by (A)BC exinuclease at 4NQO-induced sites was determined by comparison of the intensity of ABC nuclease incision bands and, in a separate lane, bands created by treatment of modified DNA with 1 M piperidine for 20 min at 90°C. These samples were lyophilized, dissolved in formamide plus dyes and electrophoresed along with the (A)BC-treated and sequencing samples. Piperidine treatment results primarily in quantitative cleavage of the C8-4AQO adduct (19), and thus the intensity of the band represents the degree of modification at a particular site with this adduct.

The efficiency of incision by (A)BC exinuclease at UV photoproducts was determined by comparison of the intensity of ABC incision bands and those produced by treatment of the DNA with T4 polymerase in the absence of dNTPs, and run in parallel gel lanes. Under such conditions, this enzyme, by virtue of its powerful 3′-5′ exonuclease, quantitatively degrades double-stranded DNA until a lesion is encountered, such as a pyrimidine dimer or a (6–4) photoproduct. The intensity of the resulting band is a reflection of the degree of damage at any one site on the DNA fragment (10,11).

Densitometric Scanning. To quantitate efficiencies of incision by (A)BC excinuclease, the ABC-treated, piperidine-treated, and T4 polymerase-treated lanes of appropriately exposed autoradiographs of sequencing gels were scanned with a Biomed Instrument Laser Scanner and the peaks integrated with an Appligration program (Dynamic Solutions Corp., PA). Incision efficiencies were determined by dividing the peak weight of the (A)BC band by the band corresponding to the same adduct created by piperidine or T4 polymerase treatment.

RESULTS

(A)BC excinuclease incision of DNA modified by 4NQO

The compound 4-nitroquinoline 1-oxide is a potent carcinogen that is metabolically activated to a form that reacts with DNA to produce adducts at the N2 and C8 positions of guanine and N6 position of adenine residues. To analyze the ability of the (A)BC excinuclease to recognize and excise these lesions *in vitro*,

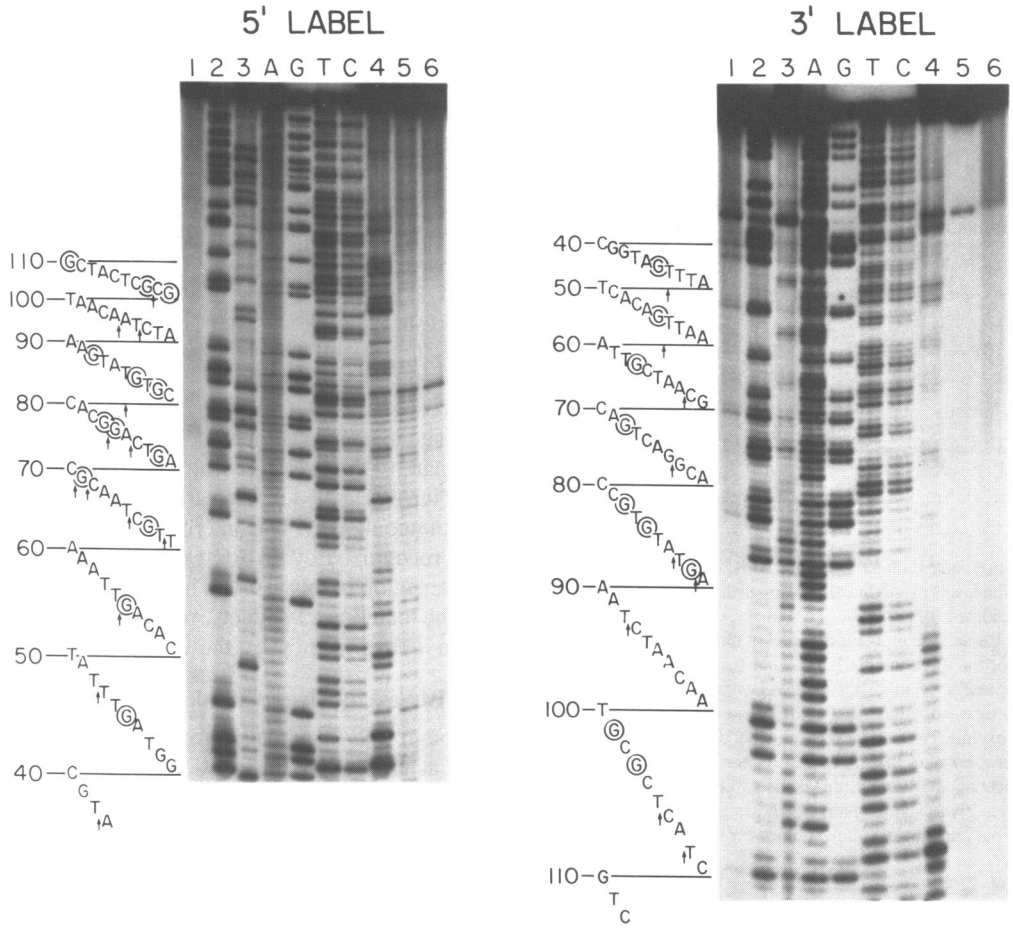

Figure 1. Incisions 5′ and 3′ to guanine 4AQO adducts by (A)BC excinuclease. Left, (Hae III-EcoR I)$_{174}$ fragment labeled 5′ at the EcoR I terminus. Right, the (EcoR I-Mae III)$_{131}$ fragment 3′ labeled at the Mae III terminus. Lane 1, DNA, no treatment; lane 2, piperidine plus heat; lane 3, (A)BC excinuclease; lanes A, G, T, C are Maxam and Gilbert sequencing reactions of A+G, G, T+C and C; lane 4, UV-DNA plus (A)BC excinuclease; lane 5, undamaged DNA plus (A)BC excinuclease; lane 6, undamaged DNA. The modified Gs (as evidenced by cleavage with piperidine) are circled; the phosphodiester bonds cleaved by (A)BC excinuclease are indicated by arrows. The numbering of bases is according to the standard pBR322 numbering system.

an activated form of the drug must be used to modify DNA. Monoacetyl-4HAQO has been shown to produce the same products as seen *in vivo* when reacted with purified DNA. We used monoacetyl-4HAQ0 made by the method of Galiegue-Zouitina *et al.* (17) to prepare modified DNA fragments containing either a unique 5′- or 3′-label on the same strand to analyze the incision pattern of (A)BC excinuclease. As seen in the left panel of Figure 1, enzyme treatment of a 5′-labeled fragment results in incisions corresponding primarily to guanine residues (lane 3). These incisions are all consistent with cleavage of the 8th phosphodiester bond 5′ to the lesion (Figure 2). C8−4AQO adducts are susceptible to cleavage by hot alkali (piperidine) which produces bands at the same position as guanine in the Maxam and Gilbert G-track (Figure 1, lane 2). This represents the extent of modification for these adducts, and thus comparison of the band with the corresponding enzyme incision band represents a measure of the efficiency of (A)BC incision (see below).

In the right panel of Figure 1, (A)BC excinuclease incisions 3′ to 4NQ0-induced adducts is examined using the same DNA sequence except with label at the 3′ terminus. Again, all the incisions made by the enzyme are at a fixed distance from G residues (compare the G lane with lane 3). The intensity of the 3′-incision by (A)BC excinuclease appears fainter than the 5′-incisions. This is not due to uncoupled incision, rather it was

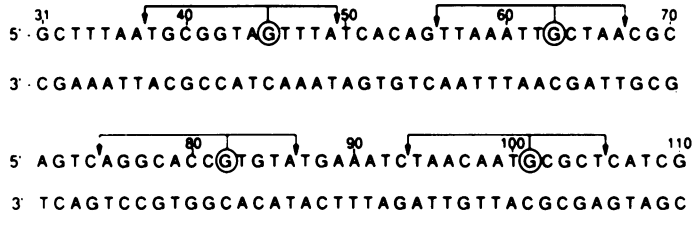

Figure 2. The Incision sites of (A)BC excinuclease relative to guanine 4AQO adducts. The incision sites were obtained from Figure 1. Only the incisions with regard to four G residues sufficiently separated from one another are shown for simplicity. The circled Gs indicate modification by 4NQO as evidenced by cleavage with piperidine. These G residues are connected to cleavage sites of (A)BC excinuclease which were interpreted to have arisen from the circled Gs.

caused by removal of the label by a 3′-5′ exonuclease contaminant present in one of the (A)BC excinuclease subunits. The 3′-incisions are all at the 5th phosphodiester bond from the adduct (Figure 2). The excised oligomer (12 bases) is thus the same as those produced by (A)BC excinuclease when excising other monoadducts (1).

The efficiency of incision by (A)BC excinuclease at 4AQO-adducts was determined by scanning of the (A)BC and piperidine lanes of the 5′-label autoradiogram and comparing the corresponding band intensities. The results are presented in panel

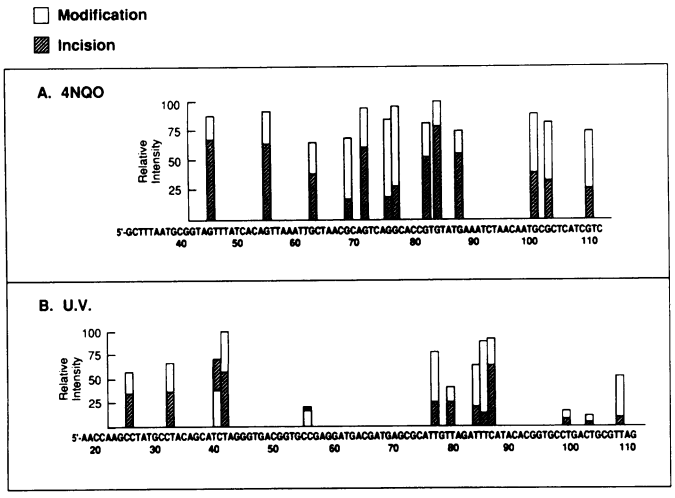

Figure 3. Effect of sequence context on incisions by (A)BC excinuclease of 4NQO or UV damaged DNA. The levels of modification were estimated by densitometric scanning of either piperidine- (4NQO), or T4 Pol 3′→5′ exonuclease- (UV photoproducts) generated bands. The units for the level of modification are arbitrary and were taken as 100 for the most intense band (G84 for 4NQO and CT42 for UV). The open boxes represent the modification level as measured by piperidine cleavage and T4 polymerase stop sites; the shaded boxes represent the extent of cleavage by (A)BC excinuclease at the 8th phosphodiester bond 5′ to the modified base. Although it appears that there is a higher frequency of incision of UV-DNA than modification at T40-C41, this is most likely an artifact resulting from formation of thymine glycols at this site which are efficiently removed by (A)BC excinuclease (23) but may not constitute a block for the 3′→5′ exonuclease of T4 DNA polymerase.

A of Figure 3. By analyzing the (A)BC incision level and piperidine incision it can be seen that generally, the extent of modification is proportional to the incision by the repair enzyme. Most adducts are repaired at about 60−70% efficiency, which is somewhat higher than that observed for other adducts. However, some lesions were repaired with lower efficiencies, around 25−40%. No apparent sequence pattern is obvious that might explain these two classes of repair efficiencies. The same adducts that show lower 5′-incision efficiency, are also not incised efficiently on the 3′-side, suggesting this is a real phenomenon and not a gel artifact. The lesions at guanine are composed of differing quantities of N2 and C8 adducts (17,19). Since N2 adducts are not susceptible to piperidine cleavage (19), but may be recognized by (A)BC excinuclease, they might contribute to the site to site variation we observe. However, we have recently prepared DNA modified almost exclusively at the C8 position and have observed repair variability similar to that presented here (data not shown). It is interesting that the N6-adenine adduct is not recognized by (A)BC excinuclease, at least not at the level of detection in this assay. This adduct comprises about 10−20% of the total 4AQO-adducts, but apparently is recognized with less than 1% efficiency by the enzyme (our detection limit).

(A)BC excinuclease incision of UV-irradiated DNA

Though the mechanism of (A)BC excinuclease action and characterization of the excision reaction was first carried out using irradiated DNA, no systematic study has been carried out measuring the efficiency of incision at pyrimidine dimers, the major photoproduct of far UV light. Using a 5′-end labeled

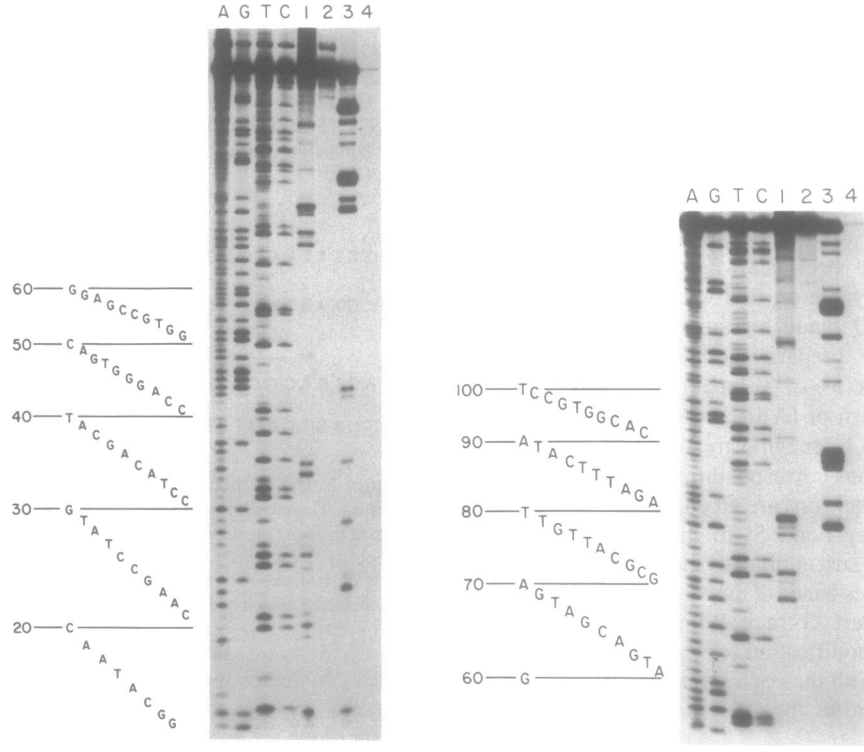

Figure 4. Incision of UV-DNA by (A)BC excinuclease. The (Hae III-EcoR I)$_{174}$ fragment with 5′ label at the Hae III site was digested by various reagents and then separated on an 8% sequencing gel. Left, short run. Right, long run. The DNA was treated with the following. Lanes A,G,T,C, Maxam-Gilbert sequencing reactions for A+G, G, T+C, and C; lane 1, (A)BC excinuclease; lane 2, no enzyme; lane 3, T4 polymerase (3′→5′ exonuclease); lane 4, undamaged DNA plus T4 polymerase. The numbering is relative to the Hae III terminus (+1) of the fragment. Note that when there are multiple lesions per fragment only one lesion 'event' can be scored by (A)BC excinuclease incision which would result in an underestimate of the incisions near the 3′ end. However, since the fragment used contains about 0.8 photoproducts per strand and the overall incision efficiency is no more than 60%, error due to 'nonscorable' incisions is minimal, though it may have contributed to the relatively low efficiency of incision at positions 103 and 108.

substrate irradiated to produce on average 1.5 photoproducts per fragment, the 5'-incisions by (A)BC excinuclease were compared to the stop sites of the 3'→5' exonuclease activity of T4 polymerase. The exonuclease stops 2 bases from a photoproduct, producing a band on sequencing gels representative of the extent of modification at that site. As seen in Figure 4, the intensity of bands due to incision by (A)BC excinuclease in lane 1 (which occur at the 8th phosphodiester bond from the lesion) are consistently lower than the corresponding bands in lane 3 representing T4 polymerase exonuclease stop sites. The overall pattern is similar to 4NQO in that the stronger the site is for formation of the adduct, the more efficient the incisions made by the repair enzyme. However, this is not true for all sites. In Figure 3, panel B, the efficiency of incision by (A)BC excinuclease is determined by comparison to the T4 exonuclease stop sites. In several cases, the efficiency is less than 20%. In others, the efficiency is quite high, such as the TC at positions 41 and 56, which presumably is due to the estimated 5-fold more efficient repair of (6–4) photoproducts (3,24) which predominate at these sites. No other sequence similarities readily explain the discrepancies where efficiency of incision is usually around 40–60%, but can be much lower. It is conceivable that possibly the more efficient sites other than TC also contain higher amounts of (6–4) photoproducts.

DISCUSSION

The major purpose of this work has been to determine the effect of sequence context on the efficiency of (A)BC excinuclease. Furthermore, the recognition and incision of 4NQO-induced lesions was demonstrated biochemically for the first time, though such adducts were known to be repaired by nucleotide excision based on genetic studies. The efficiency of incision for the two guanine adducts cannot be precisely determined yet. However, since the levels of incision at the various guanine sites generally parallels the level of modification for the C8-4AQO adduct, it appears that at least the C8-4AQO adduct is recognized and excised in the same manner as other bulky monoadducts, that is as a 12 base oligomer due to standard 5' and 3' incisions. In native DNA the C8-4AQO adduct comprises about 36% of total guanine 4AQO adducts, whereas the N2-4AQO lesion is formed at a level of about 64% (17,19). Thus, if the enzyme recognizes only the C8-4AQO adduct, the efficiency of incision for this adduct is determined directly from the ratio of band intensity of ABC excinuclease incisions and piperidine cleavage, generally about 60–70%. If the enzyme recognizes both adducts equally well, the overall efficiency would only be around 22%, whereas if only the N2-4AQO is recognized, the efficiency for excision of this lesion would be around 33%. We have recently prepared DNA containing predominantly the C8 adduct and have found incision efficiency to be about 60% (data not shown) indicating that this adduct is removed with an overall efficiency similar to that which we have found with our substrate which contains both types of adducts, suggesting that the enzyme recognizes predominantly the C8 adduct. A method has been developed recently to prepare substrate containing predominantly the N2 adduct (25). That substrate will be the subject of future studies and should make it possible to determine whether and with what efficiency the N2 adduct is recognized by the enzyme. Lastly, if the N6 adenine adduct is excised, it is at a very low level, based on the formation efficiencies reported for the various 4AQO adducts.

In general, the enzyme recognizes and excises DNA lesions in proportion to their level of modification. In most cases, this is about 60–70% for the 4AQO adducts and somewhat lower for pyrimidine dimers. The (6–4) photoproducts are also consistently repaired better than pyrimidine dimers. From the standpoint of the recognition mechanism of (A)BC excinuclease, the poor affinity of the enzyme to pyrimidine dimers compared to all other bulky adducts is quite interesting. However, little is known about the enzyme's damage recognition mechanism and no conclusion can be made at present as to why pyrimidine dimers are such a poor substrate. It is worth noting however, that while nucleotide exicision is the sole mechanism for repairing (6–4) photoproducts and other bulky adducts, pyrimidine dimers are repaired with high efficiency by DNA photolyase (1)

The observation of a class of both 4AQO monoadducts and pyrimidine dimers that are repaired relatively inefficiently is also of interest. No obvious sequence similarities arise from comparing the surrounding bases of these sites. In the case of the 4NQO-modified DNA as discussed above, mixtures of the N2 and C8 adducts of guanine certainly are produced, and the relative efficiency of repair of each of these may vary from site to site, accounting for the apparent discrepancy between the level of modification and incision.

Similarly, mixtures of dimers and (6–4) photoproducts probably occur at sequences other than TC, and the generally higher efficiency of incision of the latter by (A)BC may partially account for the variable efficiencies observed. However, as a general rule both for 4NQO adducts and for UV photoproducts the level of incision closely follows the level of modification at a given site. Thus, we conclude that, with some exceptions, (A)BC excinuclease removes adducts from B-DNA in a sequence-independent manner.

ABBREVIATIONS

4NQO, 4-nitroquinoline 1-oxide; diacetyl-4HAQO, 0,0'-diacetyl-4-(hydroxyamino)quinoline 1-oxide; monoacetyl-4HAQO, o-acetyl-4-(hydroxyamino)quinoline 1-oxide; 4AQO, 4-aminoquinoline 1-oxide; C8–4AQO, the 4AQO adduct at the C8 position of guanine; N2–4AQO, the 4AQO adduct at the N2 position of guanine.

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